

Identification of Ovotransferrin as an Acute Phase Protein in Chickens¹

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ABSTRACT Inflammation is homeostatic process associated with a variety of cellular injuries resulting from infections, toxicosis, and physical trauma. The studies on inflammation in avian species are limited. To understand the inflammation-induced changes, 4-wk-old male broiler chickens were subjected to experimental inflammation by a subcutaneous injection of croton oil (inflammatory) with changes in serum measured over time and were compared with birds treated similarly with olive oil (injected control). Croton oil treatment significantly elevated serum interleukin (IL)-6 concentrations and heterophil counts by 6 and 16 h postinjection, respectively, which returned to the basal levels of controls at 16 and 24 h, respectively. Croton oil treatment affected the serum protein profiles of chickens as assessed by SDS-PAGE and densitometric analyses. Compared with olive oil-injected or noninjected chicken sera, there were increases in the density of protein bands corresponding to molecular weights (MW) of 42, 65, 200, and 219 kDa and decreases

in bands corresponding to 49 kDa (serum albumin) and a 56-kDa protein in chickens treated with croton oil. Most of these changes were evident at 24 h and lasted through 48 h. The protein band corresponding to 65 kDa was further characterized using two-dimensional gel electrophoresis and N-terminal sequence analyses. A sequence similarity search in the Genbank database using the first 22 amino acids yielded a complete homology with chicken ovotransferrin. Western blot analysis using antichickens serum transferrin or antichickens ovotransferrin antibodies also confirmed the 65-kDa protein band to be ovotransferrin. Under nonreducing conditions, the ovotransferrin standard also showed an apparent MW corresponding to 65 kDa, like the serum transferrin. The serum ovotransferrin was found to be glycosylated using a glycoprotein stain. Although the significance of ovotransferrin in avian inflammation is not clear, these results show that it is a major acute phase protein (APP) in chickens.

(*Key words:* inflammation, chicken, ovotransferrin, acute phase protein)

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INTRODUCTION

Cellular injuries and trauma associated with infection and inflammation produce a variety of physiological changes, one of which is the alteration in the concentrations of some serum proteins called acute phase proteins (APP) (Gaby and Kushner, 1999; Kushner and Rzewnicki, 1999; Suffredini et al., 1999). Changes in the APP concentrations remain detectable until inflammation subsides in response to treatment or self-recovery. Compared to various pro-inflammatory cytokines, such as interleukins (IL) and tumor necrosis factor- α that also are elevated in response to inflammation, alterations in the levels of APP

tend to remain longer and, therefore, have better diagnostic and prognostic potential (Olfert et al., 1998).

Whereas the mammalian acute phase response is well studied, the avian acute phase response and APP are much less characterized, although in past years many studies have been published on this subject (Delers et al., 1983; Amrani et al., 1986; Grieninger et al., 1986, 1989; Hallquist and Klasing, 1994; Tohjo et al., 1995; Nakamura et al., 1998; Takahashi et al., 1998; Chamanza et al., 1999a,b). In an earlier study using lipopolysaccharide (LPS)-induced inflammation in chickens, we found significant changes in the blood concentrations of heterophils, IL-6, and serum protein profiles (Xie et al., 2000). Although the changes in the first two parameters lasted up to 24 h, the serum protein changes remained elevated through 48 h posttreatment. These changes included an

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Abbreviation Key: AP = alkaline phosphatase; APP = acute phase protein; IEF = isoelectric focusing; H+L = heavy + light chain of Ig; IL = interleukin; LPS = lipopolysaccharide; MW = molecular weight; TBST = tris-buffered saline with Tween 20.

increased concentration of a 65-kDa protein and decreased concentrations of serum albumin and a 56-kDa protein (Xie et al., 2000).

The serum APP have been used as nonspecific clinical markers of health problems in humans and other mammalian species (Gaby and Kushner, 1999); APP may provide a similar use in identifying poultry health problems. Although LPS, as a powerful systemic stimulus, has been widely used to induce inflammation, the efficacy of LPS can vary depending upon its origin, which is due to structural variations (Takahashi et al., 1995; Sunwoo et al., 1996; Berczi, 1998). In addition, the poultry species appear to be relatively resistant to LPS (Roeder et al., 1989). On the other hand, chemicals such as croton oil and turpentine oil have been used as consistent and inexpensive means to induce local inflammation with results comparable to those of LPS models (Heuertz et al., 1993; Tohjo et al., 1995).

Croton oil, the chief constituents of which are glyceride, various fatty acids, and crotonic acid, has long been used as a cathartic irritant in human and veterinary medicine (Pettit, 1977). We used subcutaneous injection of croton oil to induce inflammation to understand the acute phase response in chickens. The objective of this study was to characterize one of the serum protein bands with an apparent molecular weight (MW) of 65 kDa that showed a consistent elevation at 24 to 48 h following croton oil and LPS injection. The results showed that the 65-kDa protein, which behaves as a major acute phase protein in chickens, is the same as ovotransferrin.

MATERIALS AND METHODS

Chemicals, Reagents, and Cells

We purchased RPMI-1640 medium,³ Mark 12™ broad-range protein standard,⁴ Seebblue™ protein standard,⁴ isoelectric focusing (IEF) gels (isoelectric point, pI 3 to 10),⁴ 4 to 20% tris-glycine gels,⁴ tris/glycine/SDS running buffer (10×),⁵ recombinant human IL-6,⁶ fetal bovine serum,⁷ and reagents used for differential cell counts,⁸ rabbit anti-chicken serum transferrin antibody,⁹ rabbit anti-rat IgG (heavy + light chain of Ig; H+L) conjugated with alkaline phosphatase (AP),¹⁰ CDP-Star™ kit,¹¹ and anti-mouse IgG H+L conjugated with AP.¹² All other reagents and chemicals, including chicken ovotransferrin, albumin, and human α 1-acid glycoprotein, were purchased.¹³ An anti-chicken ovotransferrin monoclonal antibody (IgG₁ sub-

type) was developed in the USDA-ARS-Southeast Poultry Research Lab, Athens, GA.

Induction of Inflammation

One-day-old male broiler chickens (Cobb × Cobb) obtained from a local hatchery were housed in growout batteries with access to water and commercial feed ad libitum. At about 4 wk of age, the chickens were randomly assigned into five pairs of groups with five birds per treatment per time point. Inflammation was induced by injecting the birds subcutaneously in the thigh with 200 μ L of 50% croton oil in olive oil (inflammatory). Paired groups of birds were injected with an equal volume of olive oil as control for each time point. The birds were bled at 0, 6, 16, 24, and 48 h after injection. A group of untreated birds was used as basal noninjected controls. All birds were bled by cardiac puncture and killed thereafter. The protocols were according to institutional guidelines.

Hematology

Hematologic counts of EDTA-K₃-anti coagulated blood were by using a Cell-Dyne 3500 System,⁸ which included total counts of white blood cells, heterophils, monocytes, red blood cells, hemoglobin concentration, and hematocrit.

IL-6 Bioassay

Serum IL-6 level was determined as previously described (Xie et al., 2000). Briefly, 11 μ L of serum (1:2 diluted in RPMI-1640 medium) was added to 100 μ L B9 cells/well (10^5 cells/mL) and incubated at 37 C, in a CO₂ incubator for 72 h. The IL-6-dependent proliferation of B9 cells was measured using the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide as an endpoint at 570 nm. The concentrations of serum IL-6 were calculated from a standard curve using recombinant human IL-6.

Serum Protein Profiles

The serum protein concentration was measured using a BioRad dye-binding kit and bovine serum albumin as the standard. Serum protein profiles were analyzed using 10% SDS-PAGE under nonreducing conditions (Xie et al., 2000) and equal volumes of serum. The changes in serum protein profiles between injected control and inflammatory birds were compared at each time point with Mark 12™ MW marker as the standard.⁴ In some experiments, chicken ovotransferrin, chicken albumin, chicken IgG, and human α 1-acid glycoprotein were used as references. Serum was diluted 10-fold with PBS (pH 7.4) and then diluted 1:2 in a nonreducing sample buffer and boiled for 3 min. Ten microliters of each sample (equivalent to 0.5 μ L of serum) was loaded into gels and electrophoresed at 200 V for the first 10 min followed by 100 V constant

³Cellgro® Mediatech, Inc., Herndon, VA 22071.

⁴Invitrogen, Carlsbad, CA 92008.

⁵Bio-Rad Laboratories, Hercules, CA 94547.

⁶Boehringer Mannheim Co., Indianapolis, IN 46250.

⁷HyClone Laboratories, Inc., Logan, UT 84321.

⁸Abbott Diagnostics, Abbott Park, IL 60064.

⁹Accurate Chemical & Scientific Corporation, Westbury, NY 11590.

¹⁰Zymed Laboratories, Inc., San Francisco, CA 94080.

¹¹Amersham International plc, Buckinghamshire, England HP79NA.

¹²Kirkegaard & Perry Laboratory, Gaithersburg, MD 20879.

¹³Sigma Chemical Co., St. Louis, MO 63178.

until the end of separation. After staining with Coomassie brilliant blue, the gels were destained, air-dried on cellulose acetate membranes, and scanned using a Geldoc imaging densitometer.⁵

The densities of major protein bands were calculated as area \times optical density, and the relative changes in each protein band were reported as percentage changes with respect to the average of corresponding serum proteins in the noninjected, normal birds. In some cases, the gels were stained with GelCode glycoprotein stain¹⁴ according to the manufacturer's instructions.

N-terminal Sequencing

A 65-kDa protein band showed consistent elevation in inflammatory birds in the present experiment, in LPS-induced inflammation (Xie et al., 2000), and for several other infectious diseases (unpublished). To characterize this band of protein we used sera from croton oil-injected birds at 48 h posttreatment and ran a two-dimensional SDS-PAGE³ as follows. Each serum sample was diluted with an equal volume of tris-buffered saline (pH 7.4) followed by a further dilution with an equal volume of 2 \times concentrated IEF sample buffer.⁴ Five microliters of sample was then electrophoresed in IEF gels (pI 3 to 10)⁴ at 100 V constant for the first hour, 200 V for the second h, followed by 500 V for another 30 min. Individual lanes were cut and further separated according to their MW using 4 to 20% tris-glycine gels.⁴ The two-dimensional gels were then transferred to a polyvinylidene difluoride membrane⁴ and stained with Coomassie brilliant blue. The area corresponding to 65 kDa was excised and subjected to a N-terminal sequence analysis (30 cycles) using a protein sequencer (Model 473A).¹⁵ By using the first 22 amino acid sequences, a homology search was conducted in the GenBank database¹⁶ (Altschul et al., 1997), and a possible match for the sequence was identified. The N-terminal sequencing of the protein spots corresponding to 65 kDa was repeated three times using three individual samples.

Western Blot Identification of 65-kDa Protein

Serum samples from the control and inflammatory birds at 48 h postinjection were separated using 10% SDS-PAGE under nonreducing conditions as described above and were electrophoretically transferred to nitrocellulose membranes using a NOVEX transblot apparatus.⁴ Transfer was accomplished at a constant 25 V for 2 h. The blots were blocked with 5% nonfat milk powder (wt/vol) in Tris-buffered saline with 0.05% tween 20 (TBST) at room temperature for 1 h. After being washed three times with TBST solution, 5 min each, the blots were probed with

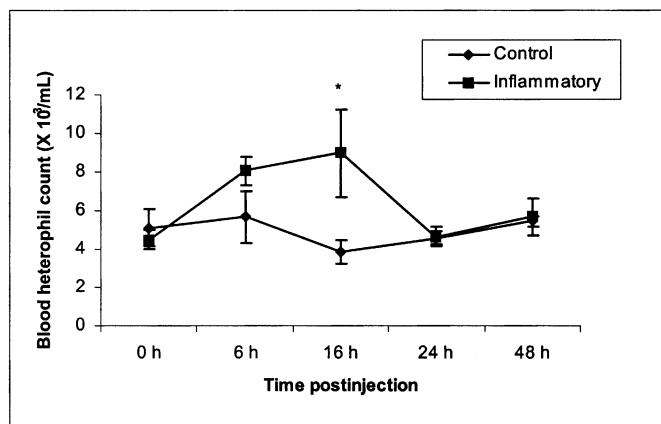


FIGURE 1. Blood heterophil counts in olive oil-treated (injected control) and croton oil-treated (inflammatory) chickens at various times postinjection. Data are expressed as mean \pm SE (n = 5). *Significant difference from controls at the corresponding time point, $P \leq 0.05$.

rabbit anti-chicken serum transferrin antibody⁸ or with nonspecific rabbit IgG¹⁰ at room temperature for an additional 1 h and washed three times, as above, 10 min each. The blots were incubated with goat anti-rabbit IgG (whole molecule) conjugated to AP¹³ at 1:100,000 dilution in TBST for 30 min. After being washed several times with excess TBST, the blots were developed using a CDP-StarTM chemiluminescence kit.¹¹ Additionally, a monoclonal anti-chicken ovotransferrin antibody was used as the primary antibody, and rabbit anti-mouse IgG (H+L) conjugated to AP¹² (1:2000) was used as the secondary antibody. Appropriate nonspecific mouse IgG¹³ was used as control. The blots were developed as above. Native and reduced forms of chicken ovotransferrin were used as references.

Statistical Analysis

Data were analyzed by Duncan's multiple-range test (SAS institute, 1988). Comparisons were made between olive oil-injected control and croton oil-injected inflammatory birds at the same time point. Significance was expressed as $P \leq 0.05$.

RESULTS

Physical Changes

Croton oil produced a bruise-like effect around the site of injection with dark yellow exudate present underneath the skin. Olive oil-injected control birds had no such changes. No differences in body weight were observed between olive oil and croton oil injection at any time.

Hematology

Compared with olive oil-injected controls, blood heterophil counts were slightly elevated in croton oil-injected inflammatory birds at 6 h, but reached a significantly high level at 16 h ($P \leq 0.05$), returning to normal levels at 24 and 48 h postinjection (Figure 1). The croton oil-treated

¹⁴Pierce, Rockford, IL 61105.

¹⁵Applied Biosystems, Foster City, CA 94404.

¹⁶Genbank Data Base: <http://www.ncbi.nlm.nih.gov/blast/>

birds had lower white blood cell concentrations ($16.8 \pm 4.6 \times 10^3/\text{mL}$; $P \leq 0.05$) at 48 h postinjection compared to noninjected ($28.6 \pm 2.3 \times 10^3/\text{mL}$) and olive oil-injected controls ($36.0 \pm 9.8 \times 10^3/\text{mL}$). These changes were not observed at other times. Neither olive nor croton oil injections had an effect on blood concentration of lymphocytes, monocytes, red blood cells, hemoglobin, or hematocrit at any of the time points.

IL-6

The IL-6 was undetected in sera from noninjected or injected control chickens at all times but increased to $0.4 \pm 0.2 \text{ ng/mL}$ ($P \leq 0.05$) in croton oil-injected birds at 6 h after injection and then returned to an undetectable level by 16 h (Figure 2).

Serum Protein Profiles

There were no statistical differences in serum protein concentrations between the olive oil injected-control and croton oil-injected inflammatory chickens (data not shown). A typical comparison of serum protein profiles at different times after injection is shown in Figure 3A. Densitometric measurement showed several changes in the protein profiles of serum in the croton oil-injected birds (Table 1). A 42-kDa protein band that was poorly stained by Coomassie blue showed strong staining with Gelcode glycoprotein stain and had migration profiles comparable to human $\alpha 1$ -acid glycoprotein in SDS-PAGE. It was undetectable in olive oil-injected control birds but was apparent by 24 h in the croton oil-injected chickens when serum albumin levels decreased. The 42-kDa protein band showed a significant increase in the croton oil-injected chickens at 24 and 48 h postinjection by using glycoprotein staining (Figure 3B). A 49-kDa protein corresponding to serum albumin showed a moderate decrease in the croton oil-injected birds during 16 to 48 h time points. A 56-kDa protein band showed a decrease after croton oil injection to reach approximately 50% of normal at 48 h postinjection ($P \leq 0.05$). A 65-kDa protein

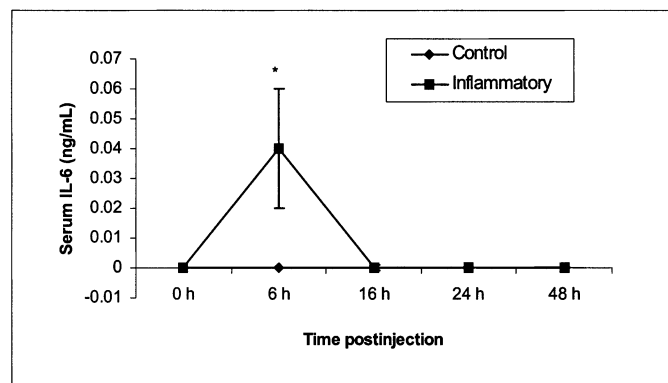
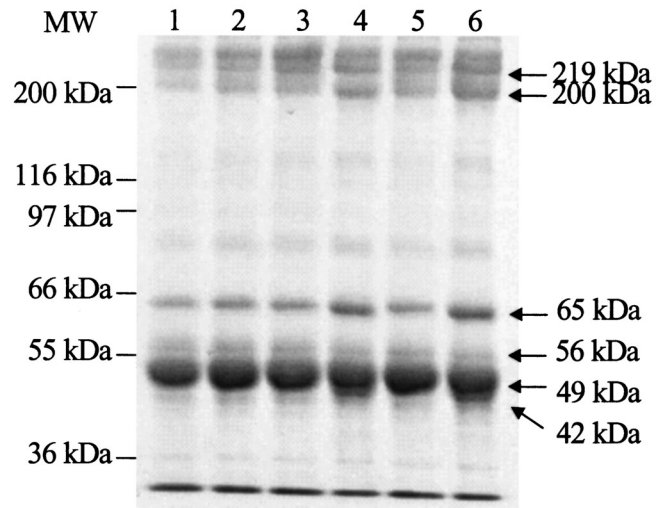


FIGURE 2. Serum interleukin (IL)-6 concentrations in olive oil- and croton oil-treated chickens at different times after injection. Data are expressed as mean \pm SE ($n = 5$). *Significant difference from controls, $P \leq 0.05$.

A



B

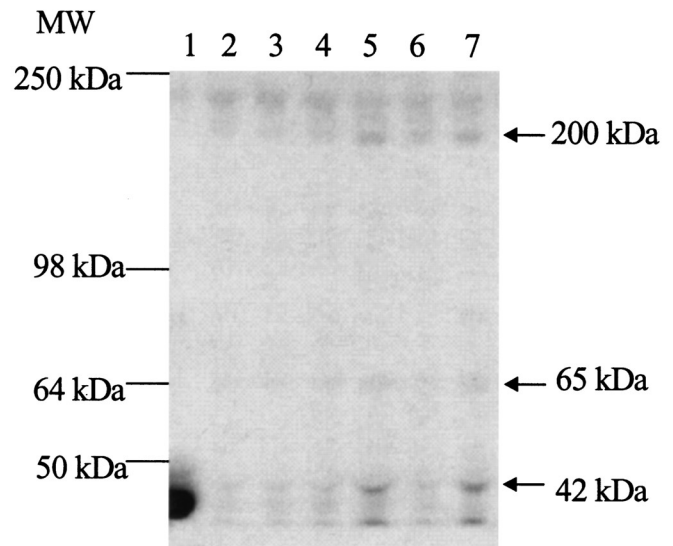


FIGURE 3. A. SDS-PAGE protein profiles of serum samples from olive oil- or croton oil-treated chickens at different times after injection. Half-microliter equivalents of pooled serum samples were electrophoresed in each lane. From left to right, Lane 1: olive oil (0 h); Lane 2: croton oil (0 h); Lane 3: olive oil (24 h); Lane 4: croton oil (24 h); Lane 5: olive oil (48 h); Lane 6: croton oil (48 h). B. Glycoprotein staining of serum samples from olive oil- or croton oil-injected chickens. Lane 1: human $\alpha 1$ -acid glycoprotein; Lane 2: olive oil (0 h); Lane 3: croton oil (0 h); Lane 4: olive oil (24 h); Lane 5: croton oil (24 h); Lane 6: olive oil (48 h); Lane 7: croton oil (48 h). MW = molecular weight.

band showed an increase in response to croton oil injection at 16 h ($P = 0.06$) and remained significantly elevated at 24 and 48 h postinjection ($P \leq 0.05$).

A protein band corresponding to 200 kDa also increased significantly at 16 h after croton oil injection ($P \leq 0.05$), which lasted through 48 h postinjection ($P \leq 0.05$). Another protein band at approximately 219 kDa tended

TABLE 1. Densitometric changes in serum proteins at different times after olive oil (injected control) and croton oil (inflammatory) treatments as compared to the noninjected control chickens¹

Molecular weight (kDa)	Group (n = 5)			Time postinjection					SEM
				0 h	6 h	16 h	24 h	48 h	
219	Noninjected control	100.0	Injected control	131.4	100.0	124.7	122.7	125.8	28.1
			Inflammatory	122.2	113.4	167.7	165.1	204.9*	
200	Noninjected control	100.0	Injected control	97.6	84.7	116.2	84.0	121.4	19.1
			Inflammatory	95.9	82.7	183.8*	159.6*	214.1*	
65	Noninjected control	100.0	Injected control	89.3	90.0	92.4	86.7	100.0	23.2
			Inflammatory	94.7	89.8	154.6	163.5*	176.8*	
56	Noninjected control	100.0	Injected control	96.0	93.1	87.6	99.3	114.3	15.9
			Inflammatory	115.0	84.3	66.7	62.4	49.6*	
49	Noninjected control	100.0	Injected control	104.0	102.3	113.9	112.2	116.7	10.0
			Inflammatory	110.0	89.2	82.3*	80.5*	71.4*	
42	Normal	BD	Injected control	BD	BD	BD	BD	BD	...
			Inflammatory	BD	BD	BD	Detectable	Detectable	

¹Percentage change of each protein band was calculated relative to the average intensity (area × optical density) of corresponding bands in noninjected normal birds. Data are presented as mean ± SEM.

²Not calculated due to being below the limits of detection (BD).

*Significant difference from controls at the same time point, $P \leq 0.05$.

to elevate after croton oil injection and had a significant increase in croton oil-injected birds at 48 h ($P \leq 0.05$). When stained with GelCode glycoprotein stain, in addition to 42-kDa protein, the 65-kDa and 200-kDa protein bands also showed mild staining (Figure 3B).

N-terminal Sequencing

The IEF profiles of serum proteins and a typical separation pattern of serum proteins in a two-dimensional gel electrophoresis is shown in Figure 4. In IEF gels, the ovotransferrin standard presented as three bands corresponding to approximate pI between 5.8 and 6.3 (Figure 4A). Three IEF bands corresponding to similar pIs were observed in serum from olive oil- and croton oil-injected birds, one of which corresponding to pI of approximately 6.3 was elevated in croton oil-injected birds between 24 to 48 h (Figure 4A). The protein with an apparent MW of 65 kDa appeared as three contiguous spots in the two-dimensional gel (Figure 4B). When sequenced, these three contiguous spots yielded an N-terminal sequence of 22 amino acids that showed a complete homology with chicken ovotransferrin (Figure 5).

Western Blot Analysis

The results of western blot analysis are shown in Figure 6. Rabbit anti-chicken serum transferrin antibodies that recognized both reduced and nonreduced forms of chicken ovotransferrin also recognized the 65 kDa bands in sera from olive oil- and croton oil-injected birds (Figure 6A). The inflammatory serum appeared have increased levels of ovotransferrin. Whereas the nonreduced form of chicken ovotransferrin standard had an apparent MW

of 65 kDa, the reduced form of chicken ovotransferrin standard showed a MW corresponding to approximately 76 kDa. The monoclonal anti-chicken ovotransferrin also recognized the 65-kDa protein in sera from olive oil- and croton oil-injected birds (Figure 6B). The control blots treated with nonspecific rabbit IgG or mouse IgG as the primary antibodies did not bind to chicken ovotransferrin standards or to the 65-kDa protein in serum (not shown).

DISCUSSION

These results show that croton oil induces an inflammatory response in chickens and has comparable characteristics of LPS-induced inflammation. These characteristics include increased blood heterophil and IL-6 concentrations and changes in serum protein profiles (Xie et al., 2000). Heterophils in relation to various stresses and infections in poultry has been noted by many investigators (Gross and Siegel, 1983; Latimer et al., 1988; Kogut et al., 1995; Maxwell and Robertson, 1998). Heterophils act as the first line of defense responsible for the containment of inflammation through phagocytic and microbicidal activities (Verdrengh and Tarkowski, 1997; Harmon, 1998). Like their mammalian counterpart neutrophils, heterophils also secrete a wide variety of enzymes, chemokines, and cytokines, which help to amplify immune responses by recruiting other cells to the site of inflammation, thereby contributing to an early resistance to infection (Kogut et al. 1998; Nikolaus et al., 1998; Rath et al., 1998; Bainton, 1999; Oliveira et al., 1999; Brandt et al., 2000). Like LPS-induced inflammation (Xie et al., 2000), there was an increase in serum concentration of IL-6 in croton oil-challenged birds. IL-6 is an important pro-inflammatory cytokine and acts along with IL-1 β and tumor

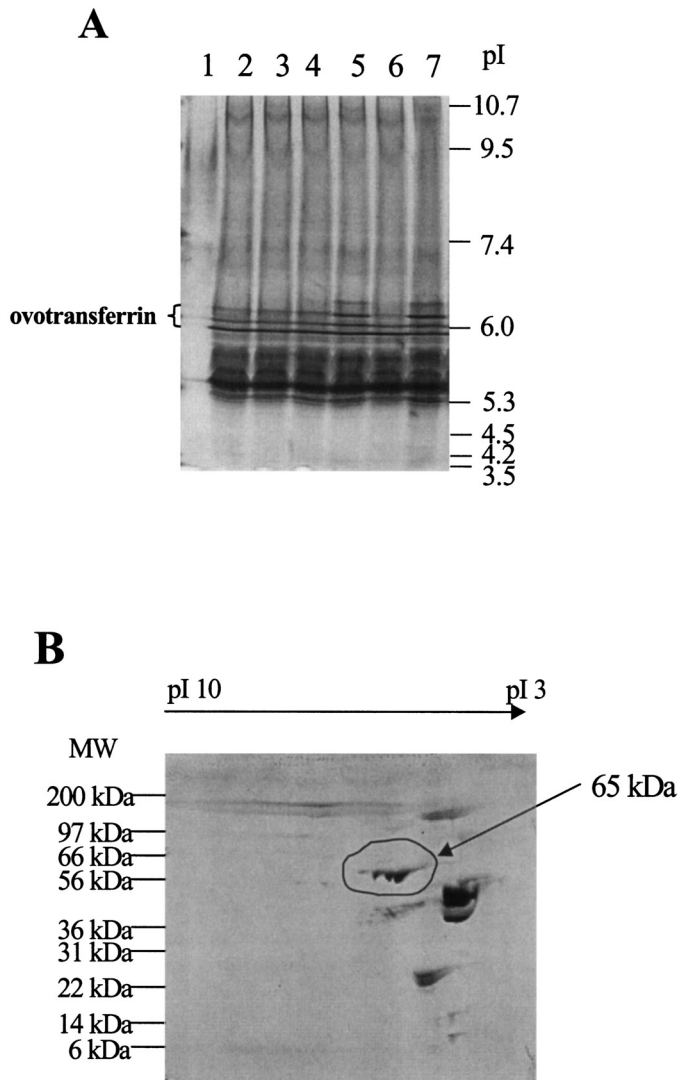


FIGURE 4. A. Isoelectric focusing profiles of serum samples from olive oil and croton oil injection at 0, 24, and 48 h after injection. Each lane represents 1.25- μ L equivalents of pooled serum samples from five birds per treatment per time point. Lane 1: chicken ovotransferrin standard; Lane 2: olive oil (0 h); Lane 3: croton oil (0 h); Lane 4: olive oil (24 h); Lane 5: croton oil (24 h); Lane 6: olive oil (48 h); Lane 7: croton oil (48 h). B. A two-dimensional gel pattern of inflammatory serum at 48 h postinjection. MW = molecular weight.

necrosis factor- α as a major regulator of synthesis and release of hepatic APP (Gauldie and Baumann, 1991; Akira and Kishimoto, 1992; Suffredini et al., 1999). IL-6 is secreted by a wide variety of cells such as endothelium, dendritic, and macrophages and mediates many other inflammation-associated symptoms (Akira and Kishimoto, 1992; Ohzato et al., 1992).

a. 65 kDa protein: APPKSVIRW_TISSPEEKK_NN
b. Chicken ovotransferrin: APPKSVIRWCTISSPEEKKCNN

FIGURE 5. N-terminal sequence comparison between the 65 kDa protein and chicken ovotransferrin. “—” represented the missing cysteine (C) in N-terminal sequencing.

Changes in the concentrations of serum APP are another major hallmark of inflammation (Gaby and Kushner, 1999; Kushner and Rzewnicki, 1999; Suffredini et al., 1999). Most APP are constitutively present in serum; however, their concentrations change discernibly in the event of inflammation and infection. These changes may include a significant elevation in concentrations of certain proteins or a decrease in others, which may vary depending upon the species (Powanda and Moyer, 1981). The changes in APP correlate with the presence and severity of inflammation and infection (Ohzato et al., 1992; Chamanza et al., 1999a,b; Kushner and Rzewnicki, 1999; Suffredini et al., 1999). In the present study, croton oil injection induced significant changes in many serum proteins, including increases in the concentrations of 42, 65, 200, and 219-kDa proteins and decreases in the concentrations of serum albumin (49 kDa) and a 56-kDa protein. We previously reported similar changes in serum albumin, a 56-kDa protein, and a 65-kDa protein in LPS-induced inflammation in male broiler chickens (Xie et al., 2000). The 42-kDa protein may be α 1-acid glycoprotein, based on its strong staining with glycoprotein stain and its migration on SDS-PAGE, which was comparable with that of human α 1-acid glycoprotein. Studies from other laboratories have suggested α 1-acid glycoprotein to be an avian APP that apparently shares immunological similarities with its mammalian counterpart (Charlwood et al., 1976; Nakamura et al., 1998; Takahashi et al., 1998).

Under normal conditions, the α 1-acid glycoprotein possibly exists in low concentrations in serum, also masked by albumin, which makes it difficult measure by densitometry; however, it becomes apparent when serum concentration of albumin is reduced during inflammation.

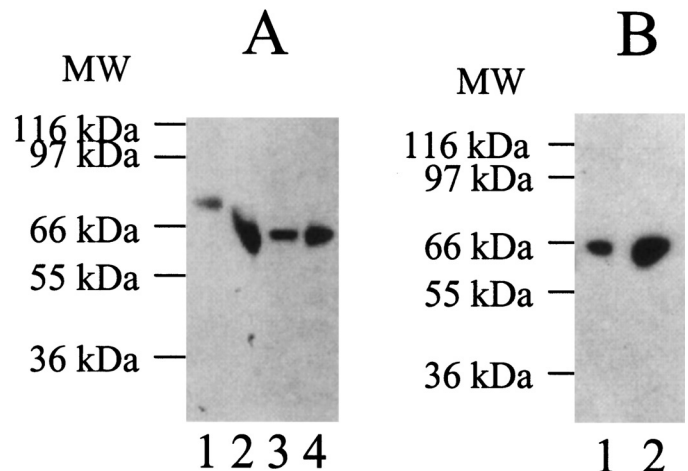


FIGURE 6. A. Western blot identification of 65-kDa protein band using a rabbit anti-chicken serum transferrin used as the primary antibody. From left to right, Lane 1: reduced chicken ovotransferrin standard; Lane 2: nonreduced chicken ovotransferrin standard; Lane 3: serum from olive oil-injected chickens at 48 h postinjection; Lane 4: serum from the croton oil-injected birds at 48 h postinjection. B. Western blot analysis of 65-kDa protein using a mouse monoclonal anti-chicken ovotransferrin as the primary antibody. From left to right, Lane 1: serum from olive oil-injected birds at 48 h postinjection; Lane 2: serum from the croton oil-injected birds at 48 h postinjection. MW = molecular weight.

By using glycoprotein staining, the changes in its levels between control and inflammatory birds were distinguishable. In the current study and in an LPS-induced inflammation model (Xie et al., 2000), a moderate reduction in serum concentration of albumin was observed, which was significantly reduced during severe and chronic inflammation (unpublished data). In this respect, birds share similarities with mammals in which serum albumin acts as a negative APP (Kushner and Rzewnicki, 1999). Similarly, a 56-kDa protein also behaved as a negative APP after croton oil and LPS injection (Xie et al., 2000); however, the nature of this protein is not known. There were a few other serum proteins with MW of 200, 219, and 65 kDa that showed an increase after croton oil injection. Several other serum proteins such as fibrinogen, hemopexin, haptoglobin, fibronectin, transferrin, mannan binding protein, and serum amyloid A have also been known to behave as avian APP and change in response to inflammatory stimuli of infectious or noninfectious origin (Delers et al., 1983; Amrani et al., 1986; Grieninger et al., 1986, 1989; Hallquist and Klasing, 1994; Tohjo et al., 1995; Chamanza et al., 1999 a,b; Nielsen et al., 1999; Lynagh and Kaiser, 2000). In the absence of authentic standards, however, it has been difficult to identify and associate these different proteins to corresponding MW bands.

Because of consistent elevations in serum concentrations of a band of protein with an apparent MW of 65 kDa in the current study and in LPS-induced inflammation (Xie et al., 2000) and the lack of any information on the identity of this protein, we characterized the 65-kDa band using two-dimensional gel electrophoresis followed by N-terminal sequence analysis. Our results showed that the 65-kDa protein is a glycoprotein with an N-terminal sequence matching to that of chicken ovotransferrin, a major egg white protein. Serum transferrin has been shown to be up-regulated during the acute phase response in chickens (Hallquist and Klasing, 1994; Tohjo et al., 1995; Chamanza et al., 1999a). However, it was not shown in these studies that serum transferrin is the same as ovotransferrin. Nonetheless, molecular sequence studies from other laboratories have shown that chicken ovotransferrin is derived from the same precursor as chicken serum transferrin with a similar amino acid sequence, except differing in the extent of glycosylation and compositions of their carbohydrate moieties (Williams, 1968; Thibodeau et al., 1978). Western blot studies with polyclonal anti-chicken serum transferrin and monoclonal anti-chicken ovotransferrin also showed that both of these antibodies recognized the 65-kDa band. However, the reported MW of ovotransferrin is ~78 kDa, which is a single polypeptide chain (Jeltsch and Chambon, 1982; Awadé et al., 1994; Lin et al., 1994).

In the current study with a Western blot, the standard ovotransferrin showed a MW of 65 kDa under nonreducing conditions similar to its serum counterpart, whereas under reducing conditions, it presented a MW of ~76 kDa. It is likely that ovotransferrin is more compact under its native conformation and migrates faster on SDS-PAGE.

Ovotransferrin is an iron-binding protein. Under capillary IEF, three distinct molecular forms of ovotransferrin have been observed, depending upon saturation with iron, i.e., iron-free ovotransferrin, monoferric ovotransferrin, and diferric ovotransferrin corresponding to pI 7.17, 6.68, and 6.09 to 6.24, respectively (Lin et al., 1994; Richards and Huang, 1997). Similar observations have also been reported by Wenn and Williams (1968) with pI slightly lower and corresponding to 6.78, 6.25, and 5.78, respectively. We also observed three bands in commercial ovotransferrin by using IEF gels (pI 3 to 10). The band corresponding to a pI of 6.3 showed higher density in serum collected from croton oil-injected birds compared to olive oil-injected controls at 24 to 48 h. Although it is difficult to directly infer whether that band corresponded to ovotransferrin, the 65-kD protein appeared to have three contiguous spots in the two-dimensional profile.

The exact function of ovotransferrin during inflammation is not known. In laying hens, ovotransferrin is synthesized under the control of estrogen (Palmiter et al., 1981) and is a major constituent of egg white. Its major physiological function, like other members of the transferrin family, is presumed to be iron transport, and its antimicrobial activities are probably related to its ability to sequester iron, an essential element for bacterial growth (Valenti et al., 1983; Jurado, 1997; Vorland, 1999; Ibrahim et al., 2000). However, unlike in mammals in which transferrin is down-regulated during inflammation (Kushner and Rzewnicki, 1999), in birds it appears to be a positive APP. Other researchers have shown the ovotransferrin to be up-regulated in fibroblasts and chondrocytes in response to inflammation and infection (Carlevaro et al., 1997; Morgan et al., 2001). In conclusion, our results show that inflammation induces changes in several serum proteins and that ovotransferrin is a positive APP in chickens.

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